DEVELOPING PRACTICAL APPROACHES TO MODIFY HEPATIC FATTY ACID PROCESSING AND LIPID MEDIATOR BIOSYNTHESIS IN DAIRY CATTLE: THE EMERGING ROLE OF LIPIDOMICS

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INTRODUCTION

Postpartum metabolic disease in dairy cattle involves the development of adipose tissue insulin resistance and hepatic lipid accretion. Although the adverse interplay between these metabolic organs is accepted, the cellular mechanisms that contribute to impaired insulin action and steatosis are not completely understood. Indeed, early lactation insulin resistance promotes adipose tissue lipolysis and dyslipidemia increases the hepatic uptake of fatty acids (**FA**). As a consequence, steatosis develops because of inadequate mitochondrial β-oxidation, enhanced triacylglycerol (**TAG**) esterification, and the cow's limited ability to export TAG within very low density lipoproteins (**VLDL**). These hallmark metabolic features of the periparturient period increase a cow's risk of obtaining a postpartum metabolic disease, and can elicit long-term consequences including immunosuppression, compromised milk production, infertility, and reduced longevity. Therefore, it is imperative that we renew our commitment to innovate and develop practical approaches that improve peripartal health. To achieve this goal, we must employ a translational dairy science approach that focuses on characterizing the biochemical mechanisms of insulin resistance and fatty liver, while simultaneously developing practical nutritional strategies that are purposefully designed to target these mechanisms. A systems-biology approach that will help us achieve this goal is the application of mass spectrometry-based lipidomics.

The advent of lipidomics has revolutionized our ability to understand lipid metabolism within the context of metabolic disease (Puri et al., 2007; Holland and Summers, 2008). Certainly, we can acknowledge the immense complexity of the bovine lipidome (i.e. the complete lipid profile within a cell, tissue, or animal). To help manage our understanding of lipid metabolism, researchers have relied on the broad classification of lipids by their shared structural attributes (e.g. mono-/di-/triacylglycerol, sphingolipid ceramide, and glycerophospholipid phosphatidylcholine (**PC**)), and dairy science has been historically limited by the unavailability of technologies to study the bioactive diversity that exists within each lipid class. One exception is our advanced understanding of nonesterified FA. We can now appreciate that FA have unique functional properties. For instance, the abilities or inabilities of saturated or polyunsaturated FA (e.g. palmitic acid, *cis*-9, *cis*-12 linoleic acid, *trans*-10, *cis*-12 conjugated linoleic acid, or docosahexaenoic acid) to modify energy metabolism and influence health are routinely characterized (Kelsey et al., 2003; Stamey et al., 2012; Loften et al., 2014). The profiling and quantification of FA by gas chromatography coupled with single or tandem mass spectrometry is a classic example of targeted lipidomics. To quantify complex lipids with

structural specificity, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is frequently employed in biomedicine. Our lab has actively utilized targeted lipidomics with LC-MS/MS to quantify 150+ complex lipids including sphingolipids (ceramides, monohexosylceramide, lactosylceramide, and sphingomyelin), fatty acylcarnitines, and glycerophospholipids (PC and phosphatidylethanolamine (**PE**)) in bovine plasma, tissues (liver, skeletal muscle, and adipose), and/or isolated lipoproteins (e.g. Davis et al., 2017a; Rico et al., 2015a,b,c, 2016, 2017a,b; Phipps et al., 2017). We have also utilized an untargeted lipidomics approach involving cutting-edge time-of-flight mass spectrometry (TOF-MS) to biochemically map 1,400+ hepatic and plasma lipids spanning 18 lipid classes in periparturient dairy cows (e.g. Saed Samii et al., 2017). Most importantly, lipidomics is being utilized to characterize the mechanisms that mediate insulin resistance and fatty liver disease in dairy cattle. The advantage of using lipidomics is that the lipidome represents the downstream product of gene transcription and translation, therefore, the lipidome is closest to the metabolic phenotype. This review will highlight the integration and progression of lipidomics within the dairy sciences. Moreover, the proposed role of sphingolipid ceramide and glycerophospholipid PC within the framework of metabolic disease will be discussed (Figure 1).

INSULIN RESISTANCE AND THE SPHINGOLIPID CERAMIDE

The mechanisms mediating insulin resistance in non-ruminants experiencing overnutrition involve the sphingolipid ceramide (Holland and Summers, 2008; Chavez and Summers, 2012). Due in part to limited mitochondrial FA oxidation, surplus saturated fatty acyl-CoA are diverted towards serine palmitoyltransferase and ceramide synthase within the de novo ceramide synthesis pathway. Alternatively, overnutrition and inflammation can promote ceramide accrual via the activation of acid sphingomyelinase. Acid sphingomyelinase controls the transformation of sphingomyelin into ceramide (Figure 1), and is activated by pro-inflammatory cytokines including tumor necrosis factor-α. The accumulation of ceramide in circulation, and skeletal muscle and adipose tissues represents a key biochemical feature of the pathophysiology that defines insulin resistance in overweight subjects experiencing lipotoxicity (Haus et al., 2009). To promote insulin resistance, ceramide can inhibit the activation of insulin receptor substrate 1 and protein kinase B to suppress insulin-stimulated glucose transporter translocation to the plasma membrane (Chavez and Summers, 2012). The inhibition of insulin signaling transduction by ceramide can trigger the protein kinase A-dependent activation of hormone sensitive lipase, thus increasing lipolysis (Mei et al., 2002). Although intracellular ceramide accumulation can promote insulin resistance, recent findings also suggest that liver-derived lipoprotein ceramide can also impair peripheral insulin action. Because hyperlipidemia, inflammation, steatosis, and insulin resistance are all linked with ceramide synthesis in non-ruminants, we are actively exploring the associative and causative roles for ceramide in dairy cattle.

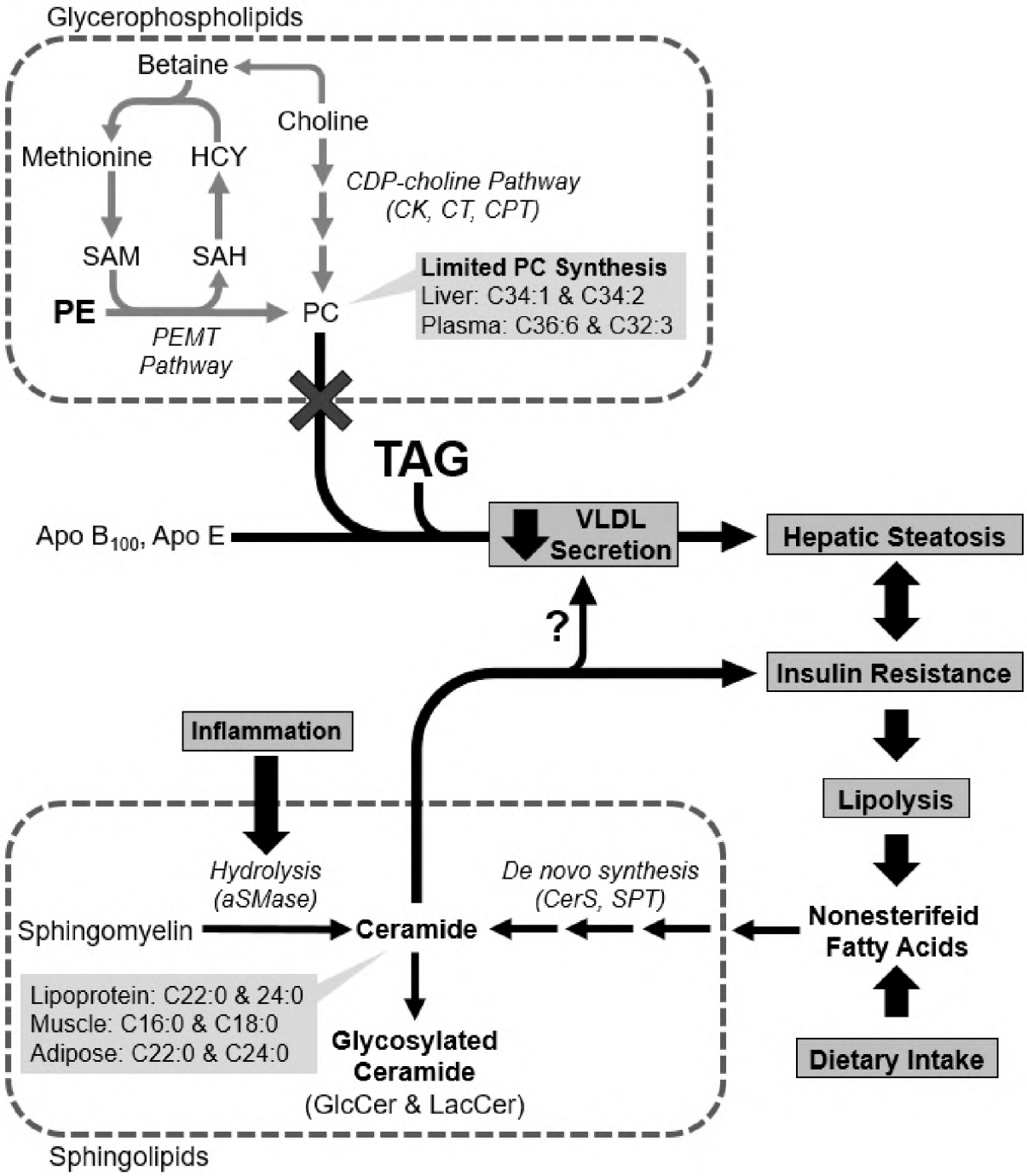

Figure 1. Proposed remodeling of the lipidome to promote insulin resistance and hepatic steatosis in the periparturient dairy cow. Metabolites and paths in bold reflect elevated levels or flux, respectively. aSMase, acid sphingomyelinase; CerS, ceramide synthase; CK, choline kinase; CPT, CDP-choline:1,2-diacylglycerol cholinephosphotransferase; CT, CTP:phosphocholine cytidylyltransferase; GlcCer, monohexosylceramide; HCY, homocysteine; LacCer, lactosylceramide; PE, phosphatidylethanolamine; PEMT; phosphatidylethanolamine *N*-methyltransferase; PC, phosphatidylcholine; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; SPT, serine palmitoyltransferase; TAG, triacylglycerol; VLDL, very low density lipoprotein.

We first demonstrated that the majority of circulating non-glycosylated and glycosylated ceramides increase in dairy cattle transitioning from gestation to lactation (Rico et al., 2015b, 2017a); however, the magnitude of increase is greater for cows with enhanced prepartum adiposity. Moreover, circulating ceramide is positively associated with the availability of nonesterified FA in plasma. These studies identified very long chain ceramides to be the most responsive (e.g. C24:0-ceramide), whereas, in a reciprocal manner, C16:0-ceramide selectively decreases postpartum. Recent data suggests that C24:0-ceramide can promote insulin resistance (Haus et al., 2009; Boon et al., 2013). In support, we demonstrated that plasma C24:0-ceramide is inversely associated with glucose clearance rates following an insulin challenge postpartum (Rico et al., 2017a); however, this relationship was not observed for circulating C16:0-ceramide. In contrast, skeletal muscle C18:0-ceramide was inversely associated with postpartum insulin-stimulated reductions of glucose. These findings suggest that the compartmentalization of specific ceramides may influence their ability to antagonize insulin action. One compartment of interest is liver tissue. Circulating ceramides are found predominantly within lipoproteins derived from liver, and lipoprotein C24:0-ceramide can inhibit insulin signaling in rodents (Boon et al., 2013). In our work, we have demonstrated that overweight cows with steatosis exhibit increased hepatic ceramide concentrations (Rico et al., 2017a), a response that develops in unison with circulating C24:0-ceramide supply. Others have demonstrated that enhanced hepatic FA uptake can increase de novo ceramide synthesis (Watt et al., 2012); however, we hypothesize that sphingomyelin hydrolysis may also contribute to ceramide synthesis in periparturient cows because they experience hepatic and systemic inflammation (Bradford et al., 2015). In support, we have demonstrated marked reductions in hepatic (unpublished) and circulating very long chain sphingomyelin concentrations (e.g. C24:0-sphingomyelin) during transition (Rico et al., 2017a), a response that develops with ceramide accrual. Although the determination of whether liver-derived lipoprotein ceramide mediates peripheral insulin action in cows is on-going, we recently developed a novel approach using fast protein liquid chromatography to isolate bovine lipoproteins for lipidomic evaluation (Phipps et al., 2017). We discovered that TAG-rich lipoproteins (e.g. VLDL) are enriched in C24:0-ceramide. The current goal is to characterize changes in lipoprotein ceramide in relation to peripartal insulin sensitivity, and determine whether lipoprotein ceramide causes adipocyte insulin resistance and lipolysis.

To further characterize ceramide metabolism in cows, we employed two in vivo models to induce hyperlipidemia in nonlactating, nongestating dairy cows. First, dairy cows were intravenously infused with a TAG emulsion for 16 h (Rico et al., 2015c). Second, dairy cows were nutrient-restricted for 32 h (Davis et al., 2017a). In both experiments, circulating FA and hepatic lipid deposition were enhanced by hyperlipidemia-induction. Similar to the transition cow, we observed marked elevations in circulating and hepatic ceramide concentrations. Again, C24:0-ceramide was most responsive. In TAG-infused cows, we did not observe a change in circulating sphingomyelin levels. These data suggest that de novo ceramide synthesis is the preferred route for ceramide synthesis in healthy cows. In support, we observed a significant increase in hepatic ceramide synthase 2 mRNA expression (controls C24:0-

ceramide synthesis; unpublished). In nutrient-restricted cows, only C22:0- and C24:0-ceramide were inversely related to insulin-stimulated glucose uptake.

If ceramide mediates insulin resistance in dairy cows, then we must be cognizant of diets that promote ceramide synthesis. Therefore, we focused our attention on palmitic acid feeding. Palmitic acid is substrate for serine palmitoyltransferase within the de novo ceramide synthesis pathway. First, we evaluated palmitic acid feeding in mid-lactation dairy cows versus no added fat control cows (Mathews et al., 2016; Rico et al., 2016). Palmitic acid feeding rapidly increased circulating ceramide, especially C24:0-ceramide. Additionally, palmitic acid feeding increased hepatic ceramide concentrations, and ceramides were inversely related to FA disappearance following a glucose challenge. We were also surprised to observe a gradual reduction in plasma ceramide levels as control cows advanced through lactation (138 to 201 DIM), a response that was decelerated by palmitic acid feeding. This gradual decline in ceramide concentrations developed with a progressive decrease in circulating FA and a gradual rise in plasma insulin. Later, we confirmed that palmitic acid feeding promotes ceramide accumulation in early lactation cows (versus no added fat controls; Davis et al., 2017b), and palmitic acid feeding is more effective at increasing ceramide synthesis than stearic acid feeding (Rico et al., 2017b). Most notably, across all three studies, circulating ceramide was positively correlated with milk yield. Our targeted lipidomic data suggest that ceramides may be intrinsically involved in the homeorhetic adaptation to lactation.

It is clear that ceramide is a biomarker for impaired insulin action in dairy cattle, particularly C24:0-ceramide. Developing nutritional strategies that target C24:0-ceramide synthesis has the potential to control insulin action in dairy cattle. First, decreasing ceramide synthesis during early lactation may be a means to improve insulin sensitivity and inhibit adipose tissue lipolysis. Such an approach would reduce hepatic FA uptake and minimize the prevalence of steatosis; however, we recognize the possibility that ceramide may be a fundamental promoter of nutrient partitioning and lactation. Therefore, decreasing ceramide synthesis may only be best served for cows at elevated risk for metabolic disease (e.g. cows with elevated prepartum adiposity). Second, increasing ceramide synthesis beyond peak milk production may be a means of inducing a homeorhetic response to maximize milk production. Moving forward, we need to continue to evaluate the role of dietary FA as modifiers of ceramide supply. For instance, polyunsaturated FA may not induce de novo ceramide synthesis because the pathway preferentially utilizes saturated FA substrate. We are actively testing this hypothesis using lipidomics. Additionally, our team is exploring novel nutritional approaches to specifically target the synthesis of C24:0-cearmide in mid-lactation dairy cows.

FATTY LIVER AND THE GLYCEROPHOSPHOLIPID PHOSPHATIDYLCHOLINE

Nonesterified FA are activated to fatty acyl-CoA; however, the catabolic or anabolic processing of fatty acyl-CoA in liver is influenced by the enhanced rate of FA uptake during the transition period. First, the capacity to completely oxidize palmitate to CO_2 is not enhanced during the transition from gestation to lactation (Litherland et al., 2011; McCarthy et al., 2015). In contrast, incomplete oxidation to acid-soluble products (i.e. TCA

cycle intermediates or ketones) is maximum during peak lipolytic response (Dann et al., 2006; Litherland et al., 2011). In non-ruminants with steatosis, hepatic FA influx develops with the accumulation of acylcarnitines in circulation (e.g., palmitoylcarnitine; Chen et al., 2016). Acylcarnitines are intermediates involved in the mitochondrial uptake of fatty acyl-CoA and represent biomarkers for defective hepatic mitochondrial β-oxidation (Adams et al., 2009; Schooneman et al., 2013). In support, our targeted lipidomic analyses have demonstrated increased plasma concentrations of acylcarnitines in transition cows (Rico et al., 2015a). As a consequence of increased hepatic FA uptake and inadequate oxidation, fatty acyl-CoA are partitioned towards TAG esterification which is mediated in part by the activation of acyltransferases.

A major contributing factor of postpartum steatosis is the cow's reduced capacity to export VLDL TAG from liver, relative to non-ruminants (Pullen et al., 1990). Our untargeted lipidomic approach has confirmed dramatic reductions in circulating TAG ranging from 18 to 97% (mean of 77%; d -28 to nadir d 3 postpartum) in 49 unique circulating TAG during the transition from gestation to lactation (e.g., TAG 60:1, 62:0, 56:1, 62:1, 58:1, and 56:4; Saed Samii et al., 2017). In parallel with a decrease in plasma TAG, we have observed similar reductions in 64 plasma monoalkyl-diacylglycerols (MADAG) during the peripartum (e.g. MADAG 52:1, 58:0, and 54:5). MADAG are ether-linked neutral lipids that aggregate with TAG and cholesteryl esters within hepatic lipid droplets and may be involved in ether phospholipid formation (Bartz et al., 2007). Our results suggest that MADAG may be transported from liver with TAG; however, the importance of MADAG for VLDL secretion is unknown. One possible explanation for limited VLDL export in cows is limited hepatic apolipoprotein (**Apo**) B_{100} concentrations. Apo B_{100} is required for VLDL assembly and secretion; however, the expression of hepatic Apo B_{100} mRNA as well as circulating Apo B_{100} concentrations decrease as parturition approaches, and are inversely related to FA levels (Bernabucci et al., 2004; Bernabucci et al., 2009).

The most promising explanation for impaired VLDL TAG export is limited supply of hepatic PC, as proposed by Van den Top et al. (1996). Glycerophospholipids form a monolayer on the lipoprotein surface surrounding the hydrophobic core, and PC is the most abundant glycerophospholipid component in all lipoprotein subclasses. For example, PC comprises ~70% (mol %) of total phospholipids of rodent plasma VLDL, whereas lyso-PC (3%) and PE (4%) are representative of minor glycerophospholipid components (Ågren et al., 2005). Furthermore, reduced levels of hepatic PC impair the secretion of VLDL from the liver (Verkade et al., 1993; Fast and Vance; 1995). Although undefined in dairy cattle, the strict requirement of PC for VLDL secretion has been demonstrated in rat hepatocytes and CTP:phosphocholine cytidylyl-transferase deficient mice (Yao and Vance, 1988; Jacobs et al., 2004). Currently, no additional evidence exists for any other glycerophospholipid requirement for VLDL secretion including PE, phosphatidylserine, phosphatidylglycerol, or the corresponding lyso-phospholipids. However, PE may be required for VLDL assembly considering that nascent VLDL contain four times more PE than plasma VLDL (Hamilton and Felding, 1989). Although little is known about the contribution of PC to VLDL synthesis and secretion in cattle, we have established that circulating total PC reaches a nadir at parturition (Saed Samii et al.,

2017), and the majority of hepatic PC decrease during the transition from gestation to lactation (e.g., PC 31:1, 37:3, and 39:5; ~60% of 118 profiled; unpublished data). Moreover, we identified multiple PC that are suppressed and predicative of postpartum fatty liver disease (e.g. PC 36:6, 32:3, 34:4, 32:2, and PC 34:6; Saed Samii et al., 2017). However, we noticed that not all circulating PC species are positively correlated with plasma TAG concentrations (e.g., PC 38:2, 42:2). Additionally, counter to common belief, the levels of a select number of hepatic PC increase postpartum (e.g., PC 34:1, 34:2, 36:2; ~25% of 118 profiled). These lipidomic data highlight the complexity of the glycerophospholipidome. Moving forward, the generalization of glycerophospholipid classes must be avoided, and the diverse structure and function of PC should be emphasized. If we can identify specific PC (i.e. acyl moieties) that are most critical for VLDL assembly and secretion, we can then develop novel nutritional approaches to target these unique PC.

The synthesis of PC occurs via two independent pathways. First, the CDP-choline pathway (i.e. Kennedy pathway) requires choline and involves three enzymatic reactions controlled by choline kinase, CTP:phosphocholine cytidylyltransferase, and CDP-choline:1,2-diacylglycerol cholinephosphotransferase. Alternatively, the phosphatidylethanolamine *N*-methyltransferase (**PEMT**) pathway involves three sequential methylations of PE, and is quantitatively relevant in liver tissue where it contributes approximately 30% of total hepatic PC synthesis (Sundler and Akesson et al., 1975). The activation of PEMT is highly dependent on the transmethylation cycle, a metabolic pathway which produces the prerequisite methyl donor *S*-adenosylmethionine (**SAM**). In liver, where PC demand for VLDL assembly and secretion is high, SAM homeostasis ensures the methylation of PE to PC (Lu, 2000). To maintain PE methylation, the transmethylation cycle can be activated by methionine and betaine (trimethylglycine) which are important dietary sources of labile methyl groups. Additionally, the irreversible oxidation of choline by choline dehydrogenase and betaine aldehyde dehydrogenase generates betaine. The availability of hepatic methionine and betaine for PC synthesis can be limited during the transition from gestation to lactation (Zeisel et al., 1995); however, evidence in rodents has demonstrated that increasing hepatic supply of methionine and betaine can increase PC synthesis and VLDL secretion (Sugiyama et al., 1998; Kharbanda et al., 2007). Because increasing hepatic PC synthesis may be a means to increase bovine VLDL synthesis and export during the periparturient period, one dietary approach to increase hepatic PC synthesis is methyl donor supplementation.

Considering that choline is the precursor for the CDP-choline pathway, and choline, methionine, and betaine are all potential methyl donors, research focused on the peripartal supplementation of these nutrients has been frequent (Piepenbrink and Overton, 2003; Davidson et al., 2008; Grummer, 2008). Initial studies with rumen-protected (**RP**) choline supplementation (variable doses) during the peripartum found no effects of choline supplementation on liver TAG concentrations (Hartwell et al., 2000; Piepenbrink and Overton, 2003; Zahra et al., 2006); albeit, Zom et al. (2011) later demonstrated the hepatic TAG-lowering ability of RP choline (~15 g/d of choline) in dairy cows. Transition cows supplemented with 15 g/d of RP methionine exhibit lower plasma

FA and β-hydroxybutyrate levels, while displaying elevated circulating VLDL and Apo B_{100} concentrations (Sun et al., 2016); although these responses may be due in part to enhanced dry matter intake. Recent evidence has demonstrated the ability of RP methionine to increase methionine adenosyltransferase 1A mRNA expression within the transmethylation cycle (Osorio et al., 2014). Research investigating betaine supplementation in dairy cows is limited. Supplementing a corn-silage total mixed ration (formulated to contain limited Met) with RP betaine (45 g/d) did not improve metabolic health or milk production (Davidson et al., 2008). In contrast, supplemental anhydrous betaine (≥ 50 g/d) lowered plasma FA and β-hydroxybutyrate concentrations, and increased milk yield in mid-lactation dairy cows (Wang et al., 2010). Unfortunately, the measurement of hepatic or VLDL PC in response to methyl donor supplementation has historically not been evaluated. In an initial investigation, we utilized lipidomics to demonstrate the ability of methyl donor supplementation (100 g/d; containing 22 g/d methionine, 10 g/d of choline chloride, and 3 g/d of betaine; MecoVit®; Vetagro S.p.A.; Zang et al., 2017) to increase multiple circulating TAG in periparturient dairy cows (e.g. TAG 46:2 and 46:3; Zang et al., 2017). We are actively evaluating the efficacy of methyl donors to increase hepatic PC synthesis and VLDL export using a combination of fast protein liquid chromatography for lipoprotein isolation and targeted lipidomics for complete PC quantitation.

POTENTIAL INTERPLAY BETWEEN CERAMIDE AND PHOSPHATIDYLCHOLINE

In the context of hepatic steatosis, the potential interplay between sphingolipid and glycerophospholipid synthesis in liver deserves attention because recent data suggests that ceramide can antagonize hepatic PC synthesis. Specifically, ceramide can inhibit CTP:phosphocholine cytidylyltransferase and CDP-choline:1,2-diacylglycerol cholinephosphotransferase within the CDP-choline pathway (Bladergroen et al., 1999; Ramos et al., 2002), and inhibit hepatic methionine adenosyltransferase gene expression and activity within the transmethylation cycle (Frago et al., 2001). Because we have demonstrated increased circulating and hepatic ceramide supply in overweight cows that develop steatosis (Rico et al., 2015b, 2017a), and hepatic PC levels are significantly suppressed in postpartum cows, the potential ability of ceramide to inhibit PC synthesis in the peripartal cow should be explored. If confirmed, then ceramide accrual may negate the efficacy of methyl donors to increase PC synthesis, and dietary approaches that decrease ceramide synthesis may be a means to maximize methyl donor efficacy. These uncertainties will need to be addressed moving forward.

CONCLUSION

The use of mass spectrometry-based lipidomics has emerged as a powerful analytical tool to understand dairy cattle nutrition and metabolism. The discovery of the sphingolipid ceramide and glycerophospholipid phosphatidylcholine as respective biomarkers of insulin sensitivity and hepatic steatosis in dairy cattle represents an early effort to utilize lipidomics. Because lipidomics can generate large data sets, the refinement of bioinformatic approaches to analyze and facilitate data interpretation represents an academic and industry challenge. This challenge can be in part overcome

by adhering to hypothesis-driven research. Lastly, the continued application of lipidomics and development of nutrition-based approaches has the potential to improve cow health; however, metabolic disease lipid mediators should be actively targeted.

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